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(57) Abstract

The present invention establishes a technology to monitor cAMP changes in living cells using two GFP variants (ECFP and EYFP). The present invention describes a construct in which the gene encoding the R subunit is essentially a linker between the genes encoding ECFP and EYFP. Following cAMP binding to both cAMP binding domains (CAB) of the recombinantly expressed R subunit, the R subunit undergoes a conformational change, thereby reducing the distance between ECFP and EYFP, which is subsequently detected by fluorescence resonance energy transfer.

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FLUORESCENCE RESONANCE ENERGY TRANSFER DETECTION OF cAMP IN LIVING CELLS USING GFP VARIANTS

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates generally to the field of molecular and cellular biology. More specifically, the present invention relates to detection of cyclic AMP using fluorescent reporter construct(s).

Description of the Related Art

Fluorescence resonance energy transfer (FRET) is a process in which an excited fluorophore (the donor) transfers its excited energy to a light absorbing molecule (the acceptor). Fluorescence resonance energy transfer is a non-destructive spectroscopic method that can monitor the proximity and relative angular orientation of fluorophores in living cells.

Green fluorescent protein (GFP) is a spontaneously fluorescent protein from the jellyfish, *Aequorea victoria*. The

cDNA encoding GFP can be fused with coding sequences from a number of other proteins; such fusion proteins usually fluoresce as well as retain the biochemical function and cellular localization of the additional protein. GFP, as well as mutants of GFP with shifted wavelengths of excitation or emission, can serve as donors and acceptors for fluorescence resonance energy transfer.

CFP (Cyan) and YFP (Yellow) are color variants of GFP. CFP and YFP contain 6 and 4 mutations, respectively. They are Tyr66Tyr, Phe66Leu, Ser65Thr, Asn145Ile, Met153Thr, and Val163Ala in CFP and Ser65Gly, Val68Leu, Ser72Ala, and Thr203Tyr in YFP. Enhanced CFP (ECFP) and enhanced YFP (EYFP) are encoded by genes with human-optimized codons. ECFP is excited at 433 nm and emits at 475 nm. EYFP is excited at 523 or 488 nm and emits at 527 nm.

There have been several previous experimental applications using GFP variants in fluorescence resonance energy transfer. For example, calcium has been measured in the cytosol and organelles of living cells (1). In these experiments, calmodulin was linked to the calmodulin-binding peptide, M13, and cloned between the genes encoding the flourophores, GFP and BFP. When Ca^{2+} bound to calmodulin, calmodulin wrapped around the M13 domain. This conformational change shortened the distance between the two fluorophore variants, thereby increasing the fluorescence resonance energy transfer. In another set of experiments, protease activity was measured *in vitro* (2). Two GFP variants were separated by a 20 amino acid flexible peptide linker that contained a Factor Xa protease site. Fluorescence resonance energy transfer gradually decreased over time due to cleavage of the peptide linker with Factor Xa, and fluorescence

resonance energy transfer was undetectable when cleavage of the linker was 100%. In yet another application, protein-protein interactions were detected in living cells (3). The Bcl-2 and Bax proteins are involved in apoptosis. The genes encoding these proteins were each fused to different variants of GFP, and then co-expressed in the same cells. Fluorescence resonance energy transfer was observed in a single intact cell, indicating that an interaction between Bcl-2 and Bax could be detected.

cAMP is an important second messenger in signal transduction pathway. Two regulatory (R) and two catalytic (C) subunits comprise the cAMP-dependent protein kinase. When cAMP binds to the R subunits, the C subunits dissociate and continue to phosphorylate other proteins. In additional experiments (4), C subunits were labeled with fluorescein isothiocyanate (FITC) and R subunits were labeled with tetramethylrhodamine isothiocyanate (Rhodamine). In the holoenzyme (C_2R_2), the dyes were close enough so that excitation of the donor (FITC) resulted in detectable emission from the acceptor (Rh) as a result of fluorescence resonance energy transfer. When cAMP bound to the R subunits, the C subunits were dissociated, thereby increasing the distance of donor-acceptor molecules to infinity and preventing fluorescence resonance energy transfer. The main disadvantage of the above-described technology is that both subunits have to be labeled with different dyes and microinjected into the cells.

The prior art is deficient in a single fluorescing reporter construct that detects cAMP levels *in vivo*. The present invention fulfills this long-standing need and desire in the art.

SUMMARY OF THE INVENTION

Because cAMP is an important second messenger in signal transduction pathways, a technology that can detect cAMP is crucial for drug screening. The present invention establishes a technology to monitor cAMP changes in living cells using two GFP variants (ECFP and EYFP). Since proteins can be tagged by GFP or one of its mutants and retain functional activity following expression, the present invention establishes a technology to monitor cAMP changes in living cells. The present invention is an improvement over previous technology because only the R subunit of cAMP-dependent protein kinase, containing two cAMP binding domains, need be labeled. The present invention describes a construct in which the gene encoding the R subunit is essentially a linker between the genes encoding ECFP and EYFP. Following cAMP binding to both cAMP binding domains (CAB) of the recombinantly expressed R subunit, the R subunit undergoes a conformational change, thereby reducing the distance between ECFP and EYFP and allowing detection by fluorescence resonance energy transfer.

One object of the present invention is to provide a single construct by which cAMP levels can be detected readily *in vivo*.

In an embodiment of the present invention, there is provided a reporter construct for monitoring cAMP levels, comprising: a) a fluorophore; b) linker DNA, comprising one or more cAMP binding (CAB) sites; and c) a light absorbing molecule.

This invention further embodies a recombinant DNA molecule encoding the reporter construct and a kit comprising the construct.

In another embodiment of the present invention, there is provided a reporter construct for monitoring cAMP levels, comprising: a) ECFP; b) linker DNA, comprising two cAMP binding (CAB) sites; and c) EYFP. This embodiment further comprises a recombinant DNA molecule, with a specific embodiment having the sequence shown in SEQ ID No. 1.

In yet another embodiment of the present invention, there is provided a method of monitoring cAMP levels in a medium, comprising the steps of: a) combining a reporter construct comprising: 1) a fluorophore; 2) linker DNA, comprising one or more cAMP binding (CAB) sites; and 3) a light absorbing molecule, with an acceptable medium to produce reporter-containing medium; b) combining a control construct with the acceptable medium, thereby producing control-containing medium, wherein the control construct comprises the fluorophore, the light absorbing molecule and the linker DNA absent the cAMP binding (CAB) sites; and c) measuring fluourescence resonance energy transfer (FRET) in said reporter-containing medium and said control-containing medium, wherein a greater amount of fluourescence resonance energy transfer in said reporter-containing medium than in said control-containing medium indicates a greater amount of cAMP in said reporter-containing medium than in said control-containing medium, wherein a lesser amount of fluourescence resonance energy transfer in said reporter-containing medium than in said control-containing medium indicates a lesser amount of cAMP in said reporter-containing medium than in said control-containing medium. This

embodiment may further comprise the steps of: d) contacting the reporter-containing medium with a stimulus; and e) measuring fluourescence resonance energy transfer in the reporter-containing medium prior to and following contact with the stimulus, wherein a greater amount of fluourescence resonance energy transfer in the reporter-containing medium following contact with the stimulus than prior to contact with the stimulus indicates an induction of cAMP levels in response to the stimulus, wherein a lesser amount of fluourescence resonance energy transfer following contact with the stimulus than prior to contact with the stimulus indicates an inhibition of cAMP levels in response to the stimulus.

Other and further aspects, features, and advantages of the present invention will be apparent from the following description of the presently preferred embodiments of the invention. These embodiments are given for the purpose of disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

So that the matter in which the above-recited features, advantages and objects of the invention, as well as others which will become clear, are attained and can be understood in detail, more particular descriptions of the invention briefly summarized above may be had by reference to certain embodiments thereof which are illustrated in the appended drawings. These drawings form a part of the specification. It is to be noted, however, that

the appended drawings illustrate preferred embodiments of the invention and therefore are not to be considered limiting in their scope.

Figure 1 shows a schematic of the present invention demonstrating *in vivo* monitoring of cAMP.

Figure 2 shows the sequence of pECFP-CAB-EYFP.

DETAILED DESCRIPTION OF THE INVENTION

Because cAMP is an important second messenger in multiple signal transduction pathways, a technology that can detect cAMP is crucial for drug screening. The present invention establishes a technology to monitor cAMP changes in living cells using two GFP variants, ECFP and EYFP. The present invention is an improvement over the prior art because only the R subunit of cAMP-dependent protein kinase, containing two cAMP binding domains, need be labeled. The present invention describes a construct in which the gene encoding the R subunit is essentially a linker between the genes encoding ECFP and EYFP. Following cAMP binding to both cAMP binding domains (CAB) of the recombinantly expressed R subunit, the R subunit undergoes a conformational change, thereby reducing the distance between ECFP and EYFP and allowing detection by fluorescence resonance energy transfer.

The technology described herein with mutants of GFP is superior over previous reports using fluorescence resonance energy transfer because there are no substrates or enzymatic

reaction required. Furthermore, it is useful in *in vivo* applications because the compounds that induce intracellular levels of cAMP can be administered directly to cells expressing the FRET-cAMP construct, pECFP-CAB-EYFP. This construct allows high throughput screening of drugs involved in cAMP signal transduction pathways.

The present invention is directed toward a single, flourescently-labelled reporter construct to detect and monitor cAMP levels *in vivo*.

The present invention is directed towards a reporter construct for monitoring cAMP levels, comprising: a) a fluorophore; b) linker DNA, comprising one or more cAMP binding (CAB) sites; and c) a light absorbing molecule. Preferably, the flourophore is selected from the group consisting of ECFP and EGFP and the light absorbing molecule is selected from the group consisting of EYFP and EBFP. The present invention further embodies a recombinant DNA molecule encoding the reporter construct and a kit comprising the construct.

One embodiment of the present invention is specifically directed toward a reporter construct for monitoring cAMP levels comprising: a) ECFP; b) linker DNA, comprising two cAMP binding (CAB) sites; and c) EYFP. Preferably, a recombinant DNA molecule comprising this construct would have the sequence shown in SEQ ID No. 1.

The present invention is further directed to a method of monitoring cAMP levels in a medium, comprising the steps of: a) combining a reporter construct comprising: 1) a fluorophore; 2) linker DNA, comprising one or more cAMP binding (CAB) sites; and

3) a light absorbing molecule, with an acceptable medium to produce reporter-containing medium; b) combining a control construct with the acceptable medium, thereby producing control-containing medium, wherein the control construct comprises the fluorophore, the light absorbing molecule and the linker DNA absent the cAMP binding (CAB) sites; and c) measuring fluourescence resonance energy transfer (FRET) in the reporter-containing medium and the control-containing medium. A greater amount of fluourescence resonance energy transfer in the reporter-containing medium than in the control-containing medium indicates a greater amount of cAMP in the reporter-containing medium than in the control-containing medium, while a lesser amount of fluourescence resonance energy transfer in the reporter-containing medium than in the control medium indicates a lesser amount of cAMP in the reporter-containing medium than in the control medium.

This embodiment of the method of the present invention may further comprise the steps of: d) contacting the reporter-containing medium with a stimulus; and e) measuring fluourescence resonance energy transfer in the reporter-containing medium prior to and following contact with the stimulus. A greater amount of fluourescence resonance energy transfer in the reporter-containing medium following contact with the stimulus than prior to contact with the stimulus indicates an induction of cAMP levels in response to the stimulus. In contrast, a lesser amount of fluourescence resonance energy transfer following contact with the stimulus than prior to contact with the stimulus indicates an inhibition of cAMP levels in response to the stimulus. A representative stimulus may include pharmaceutical

drugs, known inducers of cAMP or cAMP pathways, known inhibitors of cAMP or cAMP pathways, putative inducers of cAMP or cAMP pathways or putative inhibitors of cAMP or cAMP pathways. Generally, fluorescence resonance energy transfer may be measured by CCD cameras, FACS or by fluorometry.

As used herein, "reporter" refers to a molecule (usually a protein) that is expressed in response to or as a result of a particular biological or molecular event.

As used herein, the term "fluorophore" refers to the fluroescent group in a molecule.

As used herein, the term "light absorbing molecule" refers to the fluorophore molecule which accepts energy from a donor fluorophore.

In accordance with the present invention, there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook, Fritsch & Maniatis, "Molecular Cloning: A Laboratory Manual (1982); "DNA Cloning: A Practical Approach," Volumes I and II (D.N. Glover ed. 1985); "Oligonucleotide Synthesis" (M.J. Gait ed. 1984); "Nucleic Acid Hybridization" [B.D. Hames & S.J. Higgins eds. (1985)]; "Transcription and Translation" [B.D. Hames & S.J. Higgins eds. (1984)]; "Animal Cell Culture" [R.I. Freshney, ed. (1986)]; "Immobilized Cells And Enzymes" [IRL Press, (1986)]; B. Perbal, "A Practical Guide To Molecular Cloning" (1984). Therefore, if appearing herein, the following terms shall have the definitions set out below.

A "DNA molecule" refers to the polymeric form of deoxyribonucleotides (adenine, guanine, thymine, or cytosine) in its either single stranded form, or a double-stranded helix. This term refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, *inter alia*, in linear DNA molecules (e.g., restriction fragments), viruses, plasmids, and chromosomes. In discussing the structure herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the nontranscribed strand of DNA (i.e., the strand having a sequence homologous to the mRNA).

A "vector" is a replicon, such as plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment. A "replicon" is any genetic element (e.g., plasmid, chromosome, virus) that functions as an autonomous unit of DNA replication *in vivo*; i.e., capable of replication under its own control. An "origin of replication" refers to those DNA sequences that participate in DNA synthesis. An "expression control sequence" is a DNA sequence that controls and regulates the transcription and translation of another DNA sequence. A coding sequence is "operably linked" and "under the control" of transcriptional and translational control sequences in a cell when RNA polymerase transcribes the coding sequence into mRNA, which is then translated into the protein encoded by the coding sequence.

In general, expression vectors containing promoter sequences which facilitate the efficient transcription and translation of the inserted DNA fragment are used in connection with the host. The expression vector typically contains an origin

of replication, promoter(s), terminator(s), as well as specific genes which are capable of providing phenotypic selection in transformed cells. The transformed hosts can be fermented and cultured according to means known in the art to achieve optimal cell growth.

A DNA "coding sequence" is a double-stranded DNA sequence which is transcribed and translated into a polypeptide *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (e.g., mammalian) DNA, and even synthetic DNA sequences. A polyadenylation signal and transcription termination sequence will usually be located 3' to the coding sequence. A "cDNA" is defined as copy-DNA or complementary-DNA, and is a product of a reverse transcription reaction from an mRNA transcript. An "exon" is an expressed sequence transcribed from the gene locus, whereas an "intron" is a non-expressed sequence that is from the gene locus.

Transcriptional and translational control sequences are DNA regulatory sequences, such as promoters, enhancers, polyadenylation signals, terminators, and the like, that provide for the expression of a coding sequence in a host cell. A "cis-element" is a nucleotide sequence, also termed a "consensus sequence" or "motif", that interacts with other proteins which can upregulate or downregulate expression of a specicif gene locus. A "signal sequence" can also be included with the coding sequence. This

sequence encodes a signal peptide, N-terminal to the polypeptide, that communicates to the host cell and directs the polypeptide to the appropriate cellular location. Signal sequences can be found associated with a variety of proteins native to prokaryotes and eukaryotes.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site, as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eukaryotic promoters often, but not always, contain "TATA" boxes and "CAT" boxes. Prokaryotic promoters contain Shine-Dalgarno sequences in addition to the -10 and -35 consensus sequences.

The term "oligonucleotide" is defined as a molecule comprised of two or more deoxyribonucleotides, preferably more than three. Its exact size will depend upon many factors which, in turn, depend upon the ultimate function and use of the oligonucleotide. The term "primer" as used herein refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product, which is complementary to a nucleic acid strand, is induced, i.e., in the

presence of nucleotides and an inducing agent such as a DNA polymerase and at a suitable temperature and pH. The primer may be either single-stranded or double-stranded and must be sufficiently long to prime the synthesis of the desired extension product in the presence of the inducing agent. The exact length of the primer will depend upon many factors, including temperature, source of primer and use the method. For example, for diagnostic applications, depending on the complexity of the target sequence, the oligonucleotide primer typically contains 15-25 or more nucleotides, although it may contain fewer nucleotides.

"Recombinant DNA technology" refers to techniques for uniting two heterologous DNA molecules, usually as a result of *in vitro* ligation of DNAs from different organisms. Recombinant DNA molecules are commonly produced by experiments in genetic engineering. Synonymous terms include "gene splicing", "molecular cloning" and "genetic engineering". The product of these manipulations results in a "recombinant" or "recombinant molecule".

A cell has been "transformed" or "transfected" with exogenous or heterologous DNA when such DNA has been introduced inside the cell. The transforming DNA may or may not be integrated (covalently linked) into the genome of the cell. In prokaryotes, yeast, and mammalian cells for example, the transforming DNA may be maintained on an episomal element such as a vector or plasmid. With respect to eukaryotic cells, a stably transformed cell is one in which the transforming DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell

lines or clones comprised of a population of daughter cells containing the transforming DNA. A "clone" is a population of cells derived from a single cell or ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth *in vitro* for many generations. An organism, such as a plant or animal, that has been transformed with exogenous DNA is termed "transgenic".

As used herein, the term "host" is meant to include not only prokaryotes but also eukaryotes such as yeast, plant and animal cells. A recombinant DNA molecule or gene can be used to transform a host using any of the techniques commonly known to those of ordinary skill in the art. Prokaryotic hosts may include *E. coli*, *S. tymphimurium*, *Serratia marcescens* and *Bacillus subtilis*. Eukaryotic hosts include yeasts such as *Pichia pastoris*, mammalian cells and insect cells, and more preferentially, plant cells, such as *Arabidopsis thaliana* and *Tobaccum nicotiana*.

A "heterologous" region of the DNA construct is an identifiable segment of DNA within a larger DNA molecule that is not found in association with the larger molecule in nature. Thus, when the heterologous region encodes a mammalian gene, the gene will usually be flanked by DNA that does not flank the mammalian genomic DNA in the genome of the source organism. In another example, the coding sequence is a construct where the coding sequence itself is not found in nature (e.g., a cDNA where the genomic coding sequence contains introns, or synthetic sequences having codons different than the native gene). Allelic variations or naturally-occurring mutational events do not give rise to a heterologous region of DNA as defined herein.

A standard Northern blot assay can be used to ascertain the relative amounts of mRNA in a cell or tissue obtained from plant or other transgenic tissue, in accordance with conventional Northern hybridization techniques known to those persons of ordinary skill in the art. Alternatively, a standard Southern blot assay may be used to confirm the presence and the copy number of the gene in transgenic systems, in accordance with conventional Southern hybridization techniques known to those of ordinary skill in the art. Both the Northern blot and Southern blot use a hybridization probe, e.g. radiolabelled cDNA, either containing the full-length, single stranded DNA or a fragment of the DNA sequence at least 20 (preferably at least 30, more preferably at least 50, and most preferably at least 100 consecutive nucleotides in length). The DNA hybridization probe can be labelled by any of the many different methods known to those skilled in this art.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion:

EXAMPLE 1

Construct

The cAMP binding domains (CAB) were PCR amplified using the cDNA encoding the R subunit from cAMP-dependent protein kinase (CLONTECH). Using previously constructed EYFP-N1 and ECFP-C1 vectors, pECFP-EYFP was generated, and the

sequences encoding CAB were inserted between the genes encoding ECFP and EYFP. Figure 2 shows the sequence of the pECFP-CAB-EYFP construct.

The pECFP-CAB-EYFP construct was then transfected into 293 cells with a CaP Expression Kit (CLONTECH). Expression of both the cyan and yellow colors were detected with similar intensity under a fluorescent microscope.

EXAMPLE 2

cAMP Detection *In Vivo*

To examine cAMP changes *in vivo*, fluorescence resonance energy transfer was detected using a CCD camera or FACS. pECFP-CAB-EYFP was transfected into 293 cells. After 1 day, the cells are treated with Forskolin to induce cAMP. Following high affinity cAMP binding to the CAB of the recombinantly expressed R subunit, the R subunit underwent a conformational change reducing the distance between ECFP and EYFP and allowing detection of fluorescence resonance energy transfer. The plasmid without the cAMP binding sites (pECFP-EYFP) was used as a control. Constructs containing different numbers of cAMP binding sites, thereby resulting in different levels of fluorescence, can be constructed.

EXAMPLE 3

Sequence of pECFP-CAB-EYFP

NheI...(ECFP)TACAAG...TCCGGACTCAGATCTCGAGCTCAAGCTTCGAATTCTG
CAGTCGAC...(CAB5')GACATATTTGACGCCATGTTTCCTGTCACTCACATCGGTGG
GGAAACAGTCATACAGCAAGGGAATGAAGGAGATAATTTCTATGTGATTGACCAAGGAG
AAGTAGATGTATATGTGAACGGGGAATGGGTGACCAACATCAGTGAGGGGGGAAGCTTC
GGGGAGCTGGCTCTCATCTACGGCACCCCCAGAGCGGCTACCGTGAGGGCCAAGACGGA
CCTCAAGCTCTGGGGTATCGACCGTGACAGCTACAGGCGCATCCTCATGGGAAGCACAC
TGAGGAAACGCAAGATGTATGAGGAGTTCCTCAGCAAAGTCTCCATCCTAGAATCCCTG
GAGAAGTGGGAACGCCTGACTGTAGCTGATGCCCTGGAGCCTGTGCAGTTTGAAGATGG
AGAGAAAATTGTTGTGCAGGGGGAGCCTGGAGATGACTTCTACATCATCGAGGGCACTG
CTTCAGTCCTCCAGCGACGATCCCCCAATGAGGAGTACGTGGAAGTGGGGCGCCTTGGA
CCCTCTGACTACTTTGGGGAGATTGCCCTGCTGCTGAATCGGCCCCGTGCAGCCACTGT
GGTGGCCCGGGGTCCCCTCAAGTGTGTGAAGTTAGACCGGCCTCGTTTTGAGCGTTGCC
TGGGCCCCTGCTCTGAGATCCTGAAGAGGAACATCCAGCGTTACAACAGCTTCATCTCC
CTAACTGTC(CAB3')...CGGGATCCACCGGTCGCCACC...ATGGTG(EYFP)

The following references were cited herein:

1. Miyawaki, A. et al., Nature 388 (1997).
2. Mitra, R.D. et al., Gene, 173,13-17 (1996)
3. Nahajan, N. P. et al., Nature of Biotechnology (1998)
4. Adams, S. et al., Nature 349 (1991).

Any patents or publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. Further, these patents and publications are incorporated by reference herein to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

One skilled in the art will appreciate readily that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those objects, ends and advantages inherent herein. The present examples, along with the methods, procedures, treatments, molecules, and specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope of the claims.

WHAT IS CLAIMED IS:

1. A reporter construct for monitoring cAMP levels, comprising:

a) a fluorophore;

b) linker DNA, comprising one or more cAMP binding (CAB) sites; and

c) a light absorbing molecule.

2. The construct of claim 1, wherein said fluorophore is selected from the group consisting of ECFP and EGFP.

3. The construct of claim 1, wherein said light absorbing molecule is selected from the group consisting of EYFP and EBFP.

4. The kit comprising the construct of claim 1.

5. A recombinant DNA molecule encoding the reporter construct of claim 1.

6. A reporter construct for monitoring cAMP levels, comprising:

a) ECFP;

b) linker DNA, comprising two cAMP binding (CAB) sites; and

c) EYFP.

7. A recombinant DNA molecule encoding the reporter construct of claim 6.

8. The recombinant DNA molecule of claim 7 having the sequence shown in SEQ ID No. 1.

9. A method of monitoring cAMP levels in a medium, comprising the steps of:

a) combining the reporter construct of claim 1 with a medium;

b) combining a control construct with a medium, wherein said control construct comprises said fluorophore, said light absorbing molecule and said linker DNA absent said cAMP binding (CAB) sites; and

c) measuring fluourescence resonance energy transfer in said reporter-containing medium and said control medium, wherein a greater amount of fluourescence resonance energy transfer in said reporter-containing medium than in said control medium indicates a greater amount of cAMP in said reporter-containing medium than in said control medium, wherein a lesser amount of fluourescence resonance energy transfer in said reporter-containing medium than in said control medium indicates a lesser amount of cAMP in said reporter-containing medium than in said control medium.

10. The method of claim 9, further comprising the steps of:

d) contacting said reporter-containing medium with a stimulus; and

e) measuring fluourescence resonance energy transfer in said reporter-containing medium prior to and following contact with said stimulus, wherein a greater amount of fluourescence resonance energy transfer in said reporter-containing medium following contact with said stimulus than prior to contact with said stimulus indicates an induction of cAMP levels in response to said stimulus, wherein a lesser amount of fluourescence resonance energy transfer following contact with said stimulus than prior to contact with said stimulus indicates an inhibition of cAMP levels in response to said stimulus.

11. The method of claim 9, wherein said stimulus is selected from the group consisting of pharmaceutical drugs, chemicals, known inducers of cAMP or cAMP pathways, known inhibitors of cAMP or cAMP pathways, putative inducers of cAMP or cAMP pathways and putative inhibitors of cAMP or cAMP pathways.

12. The method of claim 9, wherein said fluourescence resonance energy transfer is measured by methods selected from the group consisting of CCD camera, FACS and fluorometry.

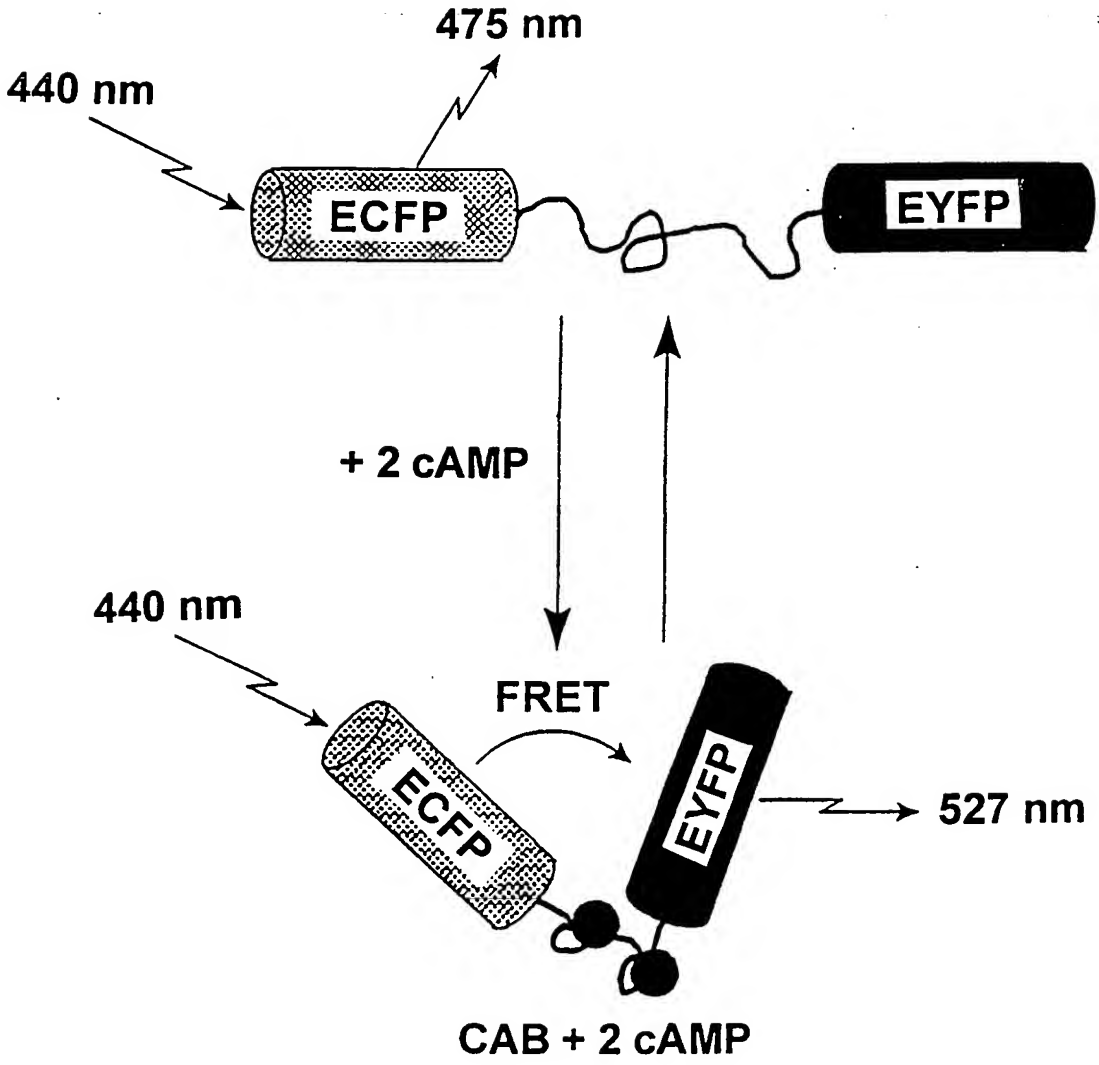
475 nm
440 nm
ECFP
EYFP
+ 2 cAMP
440 nm
FRET
ECFP
EYFP
527 nm
CAB + 2 cAMP

Fig 1

NheI...(ECFP)TACAAG...TCCGGACTCAGATCTCGAGCTCAAGCTTCGAAT
TCTGCAGTCGAC...(CAB5')GACATATTTGACGCCATGTTTCCTGTCACTC
ACATCGGTGGGGAAACAGTCATACAGCAAGGGAATGAAGGAGATAATT
TCTATGTGATTGACCAAGGAGAAGTAGATGTATATGTGAACGGGGAAT
GGGTGACCAACATCAGTGAGGGGGGAAGCTTCGGGGAGCTGGCTCTCAT
CTACGGCACCCCCAGAGCGGCTACCGTGAGGGCCAAGACGGACCTCAAG
CTCTGGGGTATCGACCGTTGACAGCTACAGGCGCATCCTCATGGGAAGC
ACACTGAGGAAACGCAAGATGTATGAGGAGTTCCTCAGCAAAGTCTCCA
TCCTAGAATCCCTGGAGAAGTGGGAACGCCTGACTGTAGCTGATGCCCT
GGAGCCTGTGCAGTTTGAAGATGGAGAGAAAATTGTTGTGCAGGGGGA
GCCTGGAGATGACTTCTACATCATCGAGGGCACTGCTTCAGTCCTCCAGC
GACGATCCCCCAATGAGGAGTACGTGGAGACCCTCTGACTACTTTGGGG
AGATTGCCCTGCTGCTGAATCGGCCCCGTGCAGCCACTGTGGTGGCCCG
GGGTCCCCTCAAGTGTGTGAAGTTAGACCGGCCTCGTTTTGAGCGTTGC
CTGGGCCCCTGCTCTGAGATCCTGAAGAGGAACATCCAGCGTTACAACA
GCTTCATCTCCCTAACTGTC(CAB3')...CGGGATCCACCGGTCGCCACC...
ATGGTG(EYFP)

Fig 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/04164

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C12Q 1/70
US CL : 435/5

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ADAMS S.R. Fluorescence ratio imaging of cyclic AMP in single cells. Letters To Nature. 21 February 1997. Vol. 349. pages 694-697, see entire document.	1-12
Y	MIYAWAKI A. Fluorescent indicators fo Ca+ based on green fluorescent proteins and calmodulin. Letters To Nature. 28 August 1997 Vol. 388. pages 882-887, see entire document.	1-12

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 22 JUNE 2000	Date of mailing of the international search report 12 JUL 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT	Authorized officer BRETT J. NELSON

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/04164

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

WEST, DIALOG, MEDLINE, EMBASE, BIOSIS, SCISEARCH
key terms: reporter construct, fluorophore, cAMP, greenm fluorescent protein, EGFP, ECFP, EYFP, EBFP, DNA molecule, recombinant, fluorescent resonance energy transfer